American Journal of Medical Genetics 64:246–251 (1996)

Long-Term Potentiation in the Hippocampus of Fragile X Knockout Mice

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To gain more insight in the physiological function of the fragile X gene (FMR1) and the mechanisms leading to fragile X syndrome, the Fmr1 gene has been inactivated in mice by gene targeting techniques. In the Morris water maze test, the Fmr1 knockout mice learn to find the hidden platform nearly as well as the control animals, but show impaired performance after the position of the platform has been modified. As malperformance in the Morris water maze test has been associated with impaired long-term potentiation (LTP), electrophysiological studies were performed in hippocampal slices of Fmr1 knockout mice to check for the presence of LTP. Judged by field extracellular excitatory postsynaptic potential recordings in the CA1 hippocampal area, Fmr1 knockout mice express LTP to a similar extent as their wild type littermates during the first 1–2 hr after high frequency stimulation. Also, short-term potentiation (STP) was similar in both types of mice.

To investigate whether Fmr1 is involved in the latter stages of LTP as an immediate early gene, we compared Fmr1 mRNA quantities on northern blots after chemical induction of seizures. A transient increase in the transcription of immediate early genes is thought to be essential for the maintenance of LTP. As no increase in Fmr1 mRNA could be detected, neither in cortex nor in total brain, during the first 2½ hr after pentylenetetrazol-induced seizures, it is unlikely that Fmr1 is an immediate early gene in mice. In conclusion, we found no evidence for a function of FMR1 in STP or LTP.

KEY WORDS: Fmr1 knockout mice, fragile X syndrome, long-term potentiation (LTP), short-term potentiation (STP), hippocampal slices, field-EPSP, CA1 area, immediate early gene

INTRODUCTION

Fragile X syndrome is one of the most commonly inherited forms of mental retardation [Oostra and Willems, 1995]. At the clinical level the fragile X syndrome is characterized by mental retardation, behavior abnormalities and hyperactivity, macroorchidism, and a peculiar face [Hagerman, 1991]. At the cytogenetic level, a fragile site is visible under folate deprived conditions at the tip of the long arm of the X chromosome (Xq27.3). At the molecular level, the fragile X syndrome is characterized by large expansions of a CGG repeat within the 5′ untranslated region of the fragile X gene (FMR1) [Verkerk et al., 1991]. Presumably as a result of repeat amplification, the promotor region of the gene becomes hypermethylated, and the transcription of the FMR1 gene is repressed [Pieretti et al., 1991]. Hence no FMR1 protein (FMRP) is present in patients [Verheij et al., 1993]. It is well established that the loss of function of the FMR1 gene causes the fragile X syndrome [Willems, 1994], as a small number of fragile X patients with deletions including the FMR1 gene have been described [for review, see Kooy et al., 1996b]. The function of the FMR1 gene product is still unknown, but the FMR1 gene has been shown to have RNA binding domains, and RNA binding properties of the FMR1 protein have been demonstrated in vitro [Ashley et al., 1993; Siomi et al., 1993, 1994; Verheij et al., 1995].

To obtain more insight in the physiological function of the FMR1 gene, a loss-of-function mutation was created in the mouse Fmr1 gene, by disrupting exon 5 of

Received for publication October 20, 1995; revision received January 2, 1996.

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the Fmr1 gene with a vector containing a neomycin gene, using homologous recombination in mouse embryonic stem cells. The knockout mice resemble human fragile X patients in having enlarged testes, hyperactivity, and demonstrating reduced performance in cognitive function tests [Bakker et al., 1994]. Cognitive functions of these mice were studied using the Morris water maze test [Morris, 1981; Morris et al., 1982]. Fmr1 knockout mice perform the test nearly as well as control mice, indicating that their spatial orientation is not severely affected by the absence of Fmr1. However, during the reversal test, when the position of the platform is altered and placed on the opposite side of the pool, knockout mice perform less well, e.g., they need more time to reach the platform than control mice [Bakker et al., 1994; Kooy et al., 1996a].

Malperformance in the Morris water maze test or the Barnes circular maze, a conceptually similar test, is also observed in other knockout mice in which genes coding for proteins involved in long-term potentiation (LTP) have been inactivated or altered, resulting in altered LTP [Grant et al., 1992; Silva et al., 1992a,b; Bach et al., 1995; Sakimura et al., 1995; Wu et al., 1995]. LTP is a long-lasting increase in synaptic strength following a strong, brief external stimulus, resulting in an increased efficiency of synaptic transmission in certain brain areas for prolonged periods. The physiological significance of LTP is still unknown, but it is generally assumed there is a link between learning, memory, and LTP. Experimentally, the increase in efficiency of synaptic transmission can be induced by high frequency stimulation to monosynaptic excitatory pathways in the hippocampus. Short-term potentiation (STP) is a related, but mechanistically different, process. It can be induced by less intense stimuli than LTP and decays within minutes, whereas LTP may last from several hours to days. A distinction can be made between LTP1 which lasts for less than 3–6 hr, LTP2 which may last up to days, and LTP3 which can last for several days up to weeks. Biochemically, LTP1, LTP2, and LTP3 are different, since LTP1 is not dependent on mRNA transcription or translation to proteins, LTP2 is dependent on the translation of pre-existing mRNA to protein, and LTP3 is dependent on the synthesis of proteins from newly transcribed mRNA [Bliss and Collingridge, 1993; Collingridge and Bliss, 1995]. A class of genes thought to be involved in LTP3 are the "immediate early" genes. Immediate early genes are hypothesized to be essential in the maintenance of LTP. This class of genes, including the transcription factors c-fos, c-jun, and zif/268, shows rapid and transient increase in expression in response to extracellular signals such as growth factors or seizures. Their mechanism of action is thought to be indirect through control of expression of a class of—unknown—genes, whose gene products bring about long term alterations in synaptic transmission [Silva and Giese, 1994; Abraham et al., 1991].

Apart from the malperformance of Fmr1 knockout mice in the Morris water maze, there are other arguments to support the hypothesis that FMR1 might be involved in LTP, as i) fragile X patients show, among other cognitive deficits, visual-spatial disabilities and impaired short-term memory [Cianchetti et al., 1991; Maes et al., 1994], ii) FMR1 expression is high in the hippocampus [Hinds et al., 1993; Devys et al., 1993; Abitbol et al., 1993; Hergersberg et al., 1995], and iii) the hippocampus of patients has been found to be of increased size in neuroradiologic studies [Reiss et al., 1995].

To investigate whether Fmr1 is involved in LTP, we performed electrophysiological studies in CA1 hippocampal slices from the Fmr1 knockout mice. Additionally, Fmr1 expression in brain after seizures was monitored, to investigate whether Fmr1 is perhaps involved in the latter stages of LTP as an immediate early gene.

MATERIALS AND METHODS

Animal Handling

The mice were kept on a 12 hr light:dark cycle with food and water supplied ad libitum. Male knockout mice [Bakker et al., 1994] and their normal male littermates were kept together in the same cages.

Slice Preparation

After ether anesthesia, the brain of Fmr1 knockout and control mice was quickly removed, and immersed in cold artificial cerebro-spinal fluid (ACSF: 134 mM NaCl, 5 mM KH_2PO_4, 1.25 mM KCl, 3 mM $CaCl_2$, 2mM $MgSO_4$, 16 mM $NaHCO_3$, and 10 mM Glucose). The hippocampus was dissected out and slices, about 400–450 μm thick, were cut perpendicularly to the long axis with a home-made chopper. Several slices were then rapidly transferred to a recording chamber (Medical Systems, NY) and placed at the interface between ACSF and an air mixture of 95% O_2 and 5% CO_2. The temperature of the perfusing fluid in the chamber was maintained at 30°C. Slices were allowed to recover for at least 90 min before the recording session.

Stimulation

Afferent fibers (Schaffer collaterals) were stimulated through a monopolar tungsten electrode placed in the stratum radiatum with a constant current of 25–150 μA for 60–70 μs with a stimulator module (Digitimer NL800). Stimulating pulses ("test pulses") were applied every 12–18 s. High frequency stimulation (HFS) was provided through the same electrode and consisted of a series of 20 pulses of 50 Hz with a duration of 60 μs, grouped in 2 or 3 trains separated by a 10 s interval as programmed on a Master8 stimulator (AMPI, Jerusalem, Israel) connected to the NL800 stimulator. The amount of current used for HFS was identical to the test pulse. However, when the test pulse was low (25–30 μA), or high (80–150 μA), a higher or lower HFS current was used, respectively.

Recording

Glass capillaries were pulled, broken back to a tip diameter of about 10 μm and filled with ACSF to be used for recording. The recording electrode was positioned in the stratum radiatum, facing the CA1 field, at about 400–550 μm distance from the stimulating electrode,

and its final position adjusted to obtain stable field extracellular excitatory postsynaptic potential recordings (field-EPSPs) of similar amplitude and slope between experiments. Signals were recorded using an Axoclamp-2A electrometer (Axon Instruments), and visualized on a memory oscilloscope. Signals were digitized at a 10 kHz frequency through a CED1401 interface and analyzed with the Sigavg program (Cambridge Electronic Design, Cambridge, UK).

Experimental Design and Analysis

To avoid any undesirable side effect, care was taken to perform standardized manipulations from one experiment to the other, and to alternate at random between the two types of mice (control and knockout mice). Recordings of ten successive responses were averaged every 3 min. Amplitude (mV) of the field-EPSP was measured by reference to the baseline level just preceding the response. Slopes of the field-EPSP (mV/s) were determined from a fixed point on the early part of the voltage deflection over a 2 ms interval. For each slice, the mean of the recordings over a period of 20 min before HFS was set at a reference value of 100%. Field-EPSPs after HFS were registered relative to this value.

RNA Isolation and Northern Blots

Normal control mice were injected subcutaneously with 60 mg/kg pentylenetetrazol in a physiological salt solution (0.9% NaCl), individually placed in empty Plexiglas cages in a quiet environment and monitored carefully [De Deyn et al., 1992]. Mice injected with physiological salt only were used as controls. Mice were decapitated after a brief ether anesthesia at 30, 60, and 150 min after the first convulsions, usually 10–15 min after drug administration. Either the whole brain or the cortex only was removed and immediately frozen in liquid nitrogen. The frozen tissues were powdered with mortar and pestle, and the tissue powder was homogenized in 1 ml TRIzol reagent (GIBCO-BRL). Total RNA was extracted according to the instructions of the manufacturer. Twenty microgram total RNA was precipitated and resuspended in 12.5 μl deionized formamide, 2.5 μl 10 × MOPS and 4 μl formaldehyde (37%), heated for 5 min at 65°C, and chilled on ice afterwards. Two and a half microliter of dye marker (50% glycerol, containing 0.1 mg/ml bromophenol blue) was added. The samples were loaded on a 1% formaldehyde-agarose gel and run in 1 × MOPS buffer for 3 hr at 100 V. After electrophoresis the gel was soaked in distilled water for 15 min, and blotted onto a Hybond N^+ nylon membrane (Amersham) for 12–16 h in 10 × SSC. The RNA was immobilized onto the membrane by baking for at least 2 hr at 80°C. The membranes were hybridized in 6 × SSC, 10 × Denhardt's, 0.1% SDS, 10% dextran sulfate with ^{32}P-dA/CTP labeled cDNA clone for 16–20 hr at 60° C. Filters were washed to 0.3 × SSC, 0.1% SDS at 65°C. The *Bgl*II fragment of clone BC22 [Verkerk et al., 1991] was used to detect Fmr1, a cloned RT-PCR product generated from the entire coding sequence of human c-fos was used to detect mouse c-fos, and cDNA pc2S15 was used to detect iduronate sulfatase (Ids).

RESULTS

Experiments were performed on 16 mice at age 20–26 weeks. From these, 17 hippocampal slices were prepared, 9 slices from 8 control mice and 8 slices from 8 knockout mice. The slices were placed in a recording chamber and the stimulating and the recording electrodes were positioned in the stratum radiatum. After a brief stimulating current through the stimulating electrode, a transient decrease of about 1 mV amplitude is registered through the second electrode, visible as an inverted peak (Fig. 1). In all experiments, the current of the stimulus was adjusted in a way that it elicited a response of 1 mV. Equal amounts of stimulating currents were needed to elicit responses of 1 mV in knockout and control mice (one-way ANOVA, $P > 0.5$).

Field-EPSP

The speed of the decrease in amplitude, or the slope of the inverted peak in Figure 1, is the parameter used to compare groups of field-EPSPs. Mean slopes recorded were 256 ± 24.6 mV/s (mean ± SE, N = 9) in the control mice, and 272 ± 31.6 (mean ± SE, N = 8) in the knockout mice. No difference in field-EPSP existed between control and knockout mice (Student's t test, $P > 0.5$).

Short-Term Potentiation

STP is a brief increase in the efficiency of synaptic transmission, that can be elicited experimentally by providing two subsequent stimulating pulses of equal current separated by a brief time interval. An increased field-EPSP after the second pulse is registered when compared to the field-EPSP after the first pulse. When two pulses are provided with 40 ms intervals, the slope of the second pulse is on average 2.1 times as steep as the first (Fig. 2). When the pulses are provided with larger time intervals, a lesser increase in slope steepness is measured, for instance only a 1.5 time increase at 140 ms intervals. STP was not significantly different between 6 control and 7 knockout mice (repeated measures ANOVA, $P > 0.5$).

Long-Term Potentiation

The field-EPSPs were recorded at regular intervals before the induction of LTP. When the response was stable for at least 20 min, LTP was induced by providing HFS, consisting of a series of pulses with a frequency of 50 Hz. After HFS, the amplitude of the field-EPSPs decreased on average 2.5 mV in contrast to 1 mV before HFS (Fig. 1), due to increased efficiency of synaptic transmission. The average slope increased to 599 ± 58 mV/s (mean ± SE, N= 9) in the controls, and to 620 ± 70 mV/s (mean ± SE, N = 8) in the knockouts. In 17 experiments, the slope of the field-EPSP remained increased for the duration of at least 80 min after HFS in 6 control and 7 knockout slices (Fig. 3). The remaining recordings were not taken into account as these had a shorter duration for technical reasons. Field-EPSPs after HFS from the knockout mice and the control mice did not differ significantly from each other (Mann Whitney U test, $P > 0.5$). Recordings of 2 control and 3

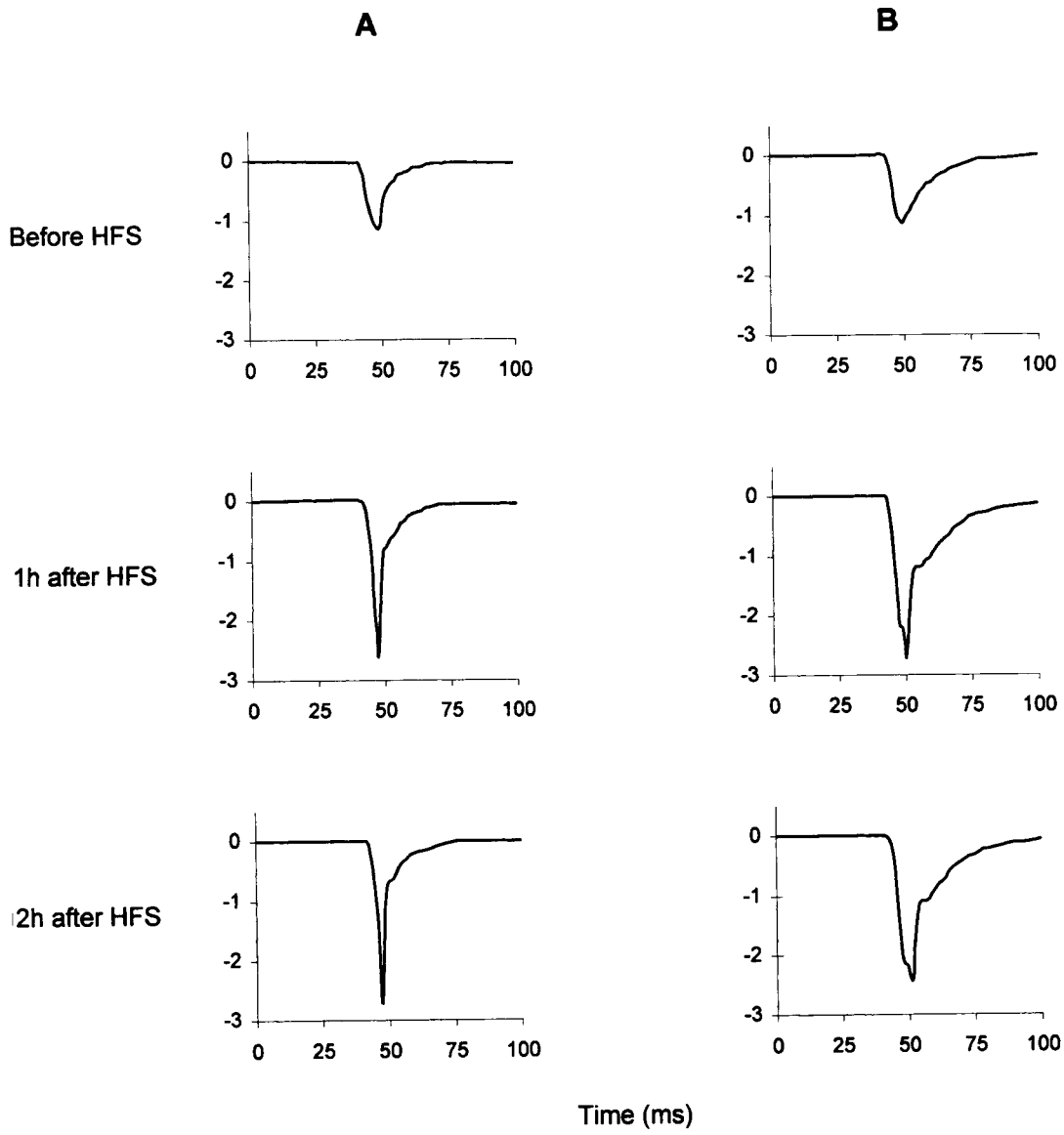

Fig. 1. Illustration of field-EPSPs before and after HFS, in control (**A**) and knockout mice (**B**). Each field-EPSP represents the average of 10 separate responses. Before HFS, a transient decrease of the field-EPSP of about 1 mV is visible. One hour after HFS, this decrease is approximately 2-2.5 mV, and remains at this level for at least 2 hours.

knockout slices could be followed for 2 hr or more, indicating that LTP remained present in both control and knockout mice for a prolonged period (data not shown).

Fmr1 Expression After the Induction of Seizures

To investigate the possible involvement of Fmr1 in LTP3 as an immediate early gene, the expression of Fmr1 mRNA was studied after injection with pentylenetetrazol to induce seizures. Seizures are known to cause a rapid transient increase in the transcription of immediate early genes such as c-fos [Morgan et al., 1987]. Northern blots were prepared from total brain or cortex mRNA and hybridized with an Fmr1 probe. An iduronate sulfatase (Ids) cDNA clone was cohybridized with the Fmr1 probe as a control probe to compare the total amounts of mRNA between the lanes. Ids is a house keeping gene coding a lysosomal enzyme which has no function in LTP. No alteration in the amount of Fmr1 mRNA could be detected in total brain or cortex up to 150 min after the first seizures, as compared to Ids (Fig. 4a). To verify whether known immediate early genes were indeed stimulated by the pentylenetetrazol inducted seizures, blots were rehybridized with c-fos. This showed no detectable c-fos mRNA before seizures and a significant amount of mRNA at 30 and 60 min after induction (Fig. 4a), which disappeared 150 min after initial convulsions (data not shown), as described previously [Morgan et al., 1987]. To ensure that the 4.5 kb band represents Fmr1 mRNA, and not one of its recently published autosomal homologues FXR1 or

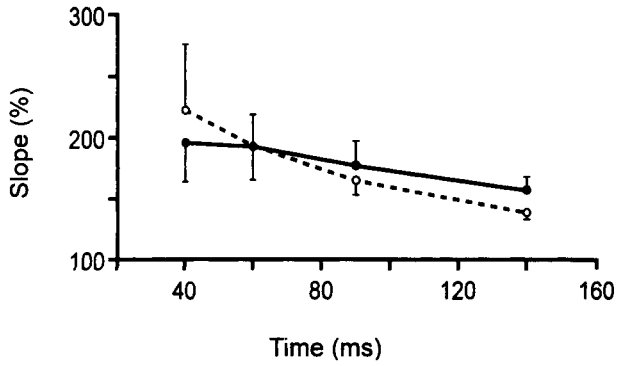

Fig. 2. Short-term potentiation in control (○) and knockout mice (●). The slope of the second stimulus, provided at fixed time intervals from the initial stimulus, is given as the percentage of the first stimulus.

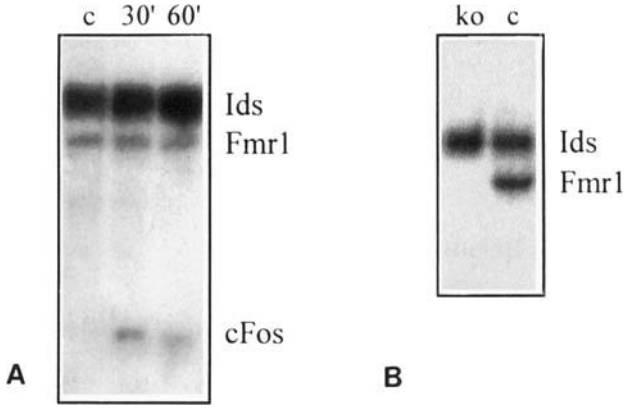

Fig. 4. (**A**) Northern blot of C57BL/6J cortex mRNA, 30 and 60 min after the first convulsions. The control mouse (c) was injected with physiological salt only. The blot was cohybridized with Fmr1 and Ids probes and rehybridized with a c-fos probe. The c-fos signal was superposed on the Fmr1 and Ids signal by computer imaging. No relative increase in intensity of Fmr1 when compared with the control probe Ids is visible, whereas c-fos expression peaks at 30–60 min after seizures. (**B**) Northern blot of total brain mRNA from a knockout (ko) and a control (c) cohybridized with an Fmr1 and an Ids probe. No Fmr1 signal is visible in the knockout.

FXR2 [Siomi et al., 1995; Zhang et al., 1995], total brain mRNA from a knockout mouse and a control mouse were co-hybridized with Fmr1 and Ids probes (Fig 4B). No Fmr1 mRNA was detectable in the knockout mice, indicating that the 4.5 kb band indeed represents Fmr1 mRNA only.

DISCUSSION

The malperformance of the Fmr1 knockout mice in the Morris water maze [Bakker et al., 1994; Kooy et al., 1996a] prompted us to investigate whether Fmr1 plays a role in the molecular mechanisms involved in LTP. We chose to study LTP in the hippocampus as the expression of Fmr1 is very high in that brain region and the hippocampus is the primary experimental model to investigate the synaptic mechanisms of learning and memory [Bliss and Collingridge, 1993; Collingridge and Bliss, 1995]. Field-EPSP recordings in the CA1 hippocampal slices of Fmr1 knockout mice showed that they are able to induce similar levels of LTP as their normal littermates. Also, induction of STP was similar, and LTP was unaltered for up to 80 min after high frequency stimulation. Therefore, it is unlikely that the Fmr1 gene is essential for the expression of pre-stimulation EPSPs, STP, and the first 1–2 hr of LTP. As our recordings lasted maximally 1–2 hr, we cannot exclude that the Fmr1 gene is involved in the latter stages of LTP. Therefore, we investigated the Fmr1 expression after induction of seizures, as immediate early genes are reported to increase transiently in expression after such an extracellular stimulus. However, no alteration in Fmr1 expression was detectable on northern blots, making it unlikely that Fmr1 is an immediate early gene involved in LTP3.

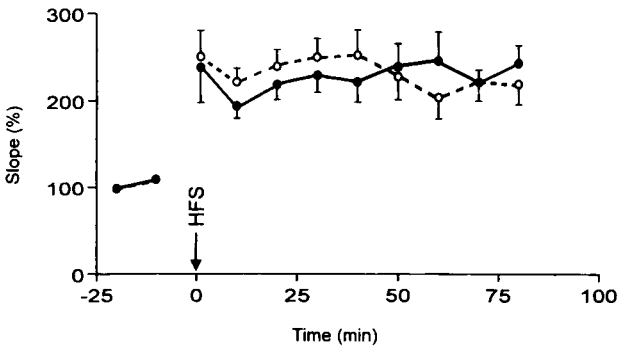

Fig. 3. Mean slope of field-EPSPs (± S.E.) of 6 control (○) and 7 knockout mice (●). The average slope of the field-EPSPs during the 20 min before HFS are set to 100%. S.E. of the field-EPSP before HFS are too small to indicate. After HFS, the average slope of the field-EPSPs has increased to 200–250% of the reference value, and remains stable for the duration of the experiments, up to 80 min.

The normal LTP in Fmr1 knockout mice is in contrast to the altered LTP found in other knockout mice models with impaired performance in the Morris water maze test. However, aberrant LTP in these models was expected as the respective inactivated or modified genes, including fyn, αCaMKII, NMDA receptor, and adenylyl cyclase, were already known or suspected to play a role in LTP [Grant et al., 1992; Silva et al., 1992a,b; Bach et al., 1995; Sakimura et al., 1995; Wu et al., 1995]. It has also to be noted that the abnormalities in the Morris water maze test of the Fmr1 knockout mice are only apparent in the reversal trial of the test, when the position of the escape platform has been changed. Morris et al. [1982] showed impaired spatial learning in rats with hippocampal lesions during the training sessions of the Morris water maze test. During the learning trials knockout mice perform equally well as the control group. Therefore, it is still unclear whether or not this really reflects a visual-spatial disability [Kooy et al., 1996a; Morris, 1984].

Thus, the visual-spatial disabilities and defects in short-term memory in fragile X patients [Maes et al., 1994; Cianchetti et al., 1991] and the impaired performance of Fmr1 knockout mice in the Morris water maze task [Bakker et al., 1994; Kooy et al., 1996a], can-

not be explained by gross abnormalities in the early phase of LTP, a key process in (spatial) learning and memory, nor by the involvement of FMR1 as an immediate early gene in LTP3. More subtle changes in LTP, or changes in the latter phases of LTP might still be present. Studies to investigate these are technically demanding, but should be undertaken to provide more insight in the role of FMR1 in learning and memory.

ACKNOWLEDGMENTS

Part of this work was supported by a concerted action of the University of Antwerp, a grant of the Association Françaises contre les Myopathies (AFM), and a grant from the Belgian National Fund for Scientific Research (NFWO) to P.J.W., and a grant from the Marguerite-Marie Delacroix foundation to E.R. J.-M.G. was supported by AC89-95/135 and FNRS/FRSM.

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